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Isolation of a Sexual Sporulation Hormone from *Aspergillus nidulans*

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Psi factor is a substance produced by *Aspergillus nidulans* that induces premature sexual sporulation. Chromatographic analysis of psi-active extracts showed that psi activity resides in several different forms. Two of the forms, psiA1 and psiB1, have been isolated and have been shown to have closely similar compositions. The most abundant form, psiA1, reacts with alcohols in acidic solution by the addition of one entire molecule of the alcohol. This reaction, which is reversible, suggests that psiA1 may be a lactone whose ring is opened by alcohol addition. At high concentration, psiA1 is antagonistic to the response exhibited by the other forms of psi, but this antagonism is lost by the alcoholic derivatives. At least one unpurified psi species can be converted to psiA1 by acid catalysis. We suggest that psiA1 may be the metabolic precursor of at least some of the other more active psi components and that this conversion during *Aspergillus* development may be part of the process that triggers sexual sporulation.

Fungi propagate principally by the formation of spores which can be either asexual or sexual. Asexual spores (conidia) are produced by mitosis of haploid nuclei, whereas sexual spores are generated by meiosis of diploid nuclei. The two haploid components of the diploid nuclei may be derived from the same strain (homothallic species) or may be obligatorily derived from different strains (heterothallic species) which are said to be of opposite mating type. Many species, including homothallic *Aspergillus nidulans*, exhibit both asexual and sexual sporulation.

When initiated from spores, fungal colonies usually grow at first by extension of their hyphal filaments (vegetative growth) and at some later time abruptly begin to make spores, either asexual, sexual, or both. The switch from vegetative growth to sporulation is accompanied by the activation of a large number of genes which can account for a considerable fraction of the expressed fungal genome. In *A. nidulans*, over 1,000 new mRNA species appear during asexual sporulation (8). A fundamental question is the nature of the signal that initiates sporulation and how this signal is transduced to the level of the gene.

In some fungal species, sexual sporulation has been shown to be mediated by specific metabolites that are secreted in very small amounts and which can reasonably be termed hormones. In the few cases for which the active agents have been characterized (4), they are terpenoids (the trisporic acids of *Mucor* species), steroids (antheridiol and oogoniols of *Achlya* species), or peptides (α and a factors of *Saccharomyces cerevisiae*).

Confluent plate cultures of *A. nidulans* normally develop in a well-defined sequence in which asexual sporulation precedes sexual sporulation. We have previously demonstrated that this organism produces a substance with potent hormonelike activity, called psi factor, that induces premature sexual sporulation, causing the two sporulation modes to overlap (3). Active extracts also cause an attenuation of asexual sporulation, which we have argued is probably a secondary effect due to competition of asexual sporulation with precocious sexual sporulation. Preliminary studies showed that psi activity is secreted into the culture medium, is extractable from the medium by organic solvents, and is sufficiently stable to allow isolation (3). In this paper we describe the resolution of psi activity into several components, the isolation and partial characterization of two of these components, and the reactivity of one of the components with alcohols, which results in a qualitative change in its biological activity.

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MATERIALS AND METHODS

Strains and media. Strain WIM-145 (*acoC193 yA2 pabaA1 ve*$^+$), whose properties have been described elsewhere (1), was used to prepare the extracts from which psi-active components were isolated. The prototrophic wild-type strain FGSC-4 was used as the indicator strain for assays of psi activity. The standard growth medium (YGT) was composed of 0.5% yeast extract (Difco Laboratories), 2% glucose, and trace elements at concentrations given previously (6). For plates, the medium was solidified with 1.5% agar. CAZ medium, which was used for large-scale culture, is essentially the same as YGT but was prepared with less expensive ingredients, with Ardamine Z (Yeast Products) replacing yeast extract and cerelose (Corn Products) replacing glucose.

Assay of psi activity. Indicator plates (9 cm in diameter and containing 25 ml of medium) were prepared by spreading 0.7 ml of medium containing 10^6 conidia of strain FGSC-4 over the surface of the agar. Samples to be assayed were dried on 4-mm filter paper disks and placed at the center of the plate after the agar surface had become dry. Psi activity was indicated by the formation around the assay disk of a yellow plaque which was observed on the back of the plate after 18 to 24 h of incubation at 37°C (see Results) (Fig. 1). The intensity of pigmentation was optimized by doubling the concentration of trace elements and halving the concentration of agar of the standard YGT medium.

Production, extraction and enrichment of psi factor. For small-scale cultures, psi activity was extracted from the culture medium by shaking the medium with ethyl acetate. For culture volumes greater than 5 liters, a more convenient alternative to solvent extraction was found to be adsorption of the activity to Amberlite XAD beads (XAD-4). The following procedure applies to a 100-liter culture. The stated

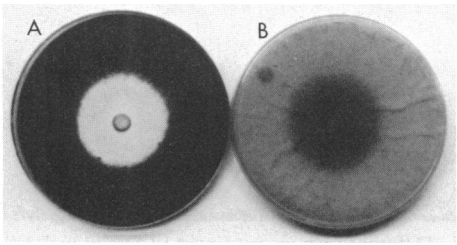

FIG. 1. Two manifestations of psi activity. (A) On the front of the plate, psi inhibited conidiation and induced premature sexual sporulation (white area). (B) On the back of the plate, psi induced a yellow pigmentation of the mycelium (dark area) before any morphological differentiation had occurred. The sample was an unfractionated ethyl acetate extract obtained from 1 ml of culture medium. See Materials and Methods for details.

yields are representative of those obtained from the processing of several batches.

CAZ medium was inoculated with 2×10^6 conidia of strain WIM145 per liter and grown at 42°C. The culture was aerated at 400 liters/min and agitated with an impeller rotating at 80 rpm. After 96 h of fermentation, the temperature was reduced to 25°C and 1 kg of Amberlite XAD beads, contained in a nylon mesh bag, was placed into the tank and swirled with the culture for 12 h.

The XAD beads, which adsorbed most of the psi activity, were eluted with methanol in a large fritted-disk funnel until the eluate was almost colorless. Evaporation of the solvent yielded 13 g of a reddish black tar. This tar was redissolved in 50 ml of methanol, and the solution was chilled overnight at −20°C. The precipitate which formed was removed by filtration and discarded, after which 250 ml of ethyl acetate was added slowly to the supernatant. After the mixture was chilled overnight at −20°C, the resulting precipitate was removed and discarded, and the solution was taken to dryness. These precipitations were repeated two times, with a reduction in the volume of the methanolic solution by one-half each time. At this point the dry weight was reduced to 4 g.

Further enrichment was achieved by gel filtration through a column of LH-20 lipophilic Sephadex (350 by 20 mm) equilibrated in methanol, with one-half of the extract applied in 5 ml of methanol per column run. The activity eluted quite reproducibly between 80 and 110 ml of eluate (measured from the start of sample application), which is just after the void volume. The active fractions, which were identified by assay of 5 μl of 10-ml fractions, were pooled, taken to dryness, and redissolved in 1 ml of methanol. To this solution, 10 ml of toluene was added slowly with stirring, and the resulting inactive precipitate was removed by centrifugation and discarded. The residue obtained upon evaporation of the toluene consisted of 0.7 g of a viscous red oil. This material, which had about 5% of the mass of the original extract, was used in the final purification steps described in Results and is called an LHP extract.

Soxhlet extraction. To extract XAD beads on a small scale without the use of methanol, a 100-ml Soxhlet apparatus (a reflux still for repeatedly extracting a solid) was used with cyclohexane as the eluting solvent. For each 50-g batch of beads, 100 ml of solvent was refluxed through the beads for 24 h. The product thus obtained represented only about 5% of the mass of material sequestered from the medium but almost all of the psi activity.

Silica gel column chromatography. Columns were packed with Kieselgel 60, 230/400 mesh (catalog no. 9385; Merck & Co., Inc.) as a slurry with the desired solvent. For samples containing 0.5 to 2 g of material, a 350-by-20-mm column was used; for samples containing less than 0.5 g of material, a 260-by-10-mm column was used. Samples were dissolved and loaded in a minimum amount of solvent and eluted with the same solvent. The standard solvent mixtures were CEE, chloroform-ethyl acetate-diethyl ether (10:4:2 [vol/vol/vol]), and benzene-ethyl acetate (4:6 [vol/vol]). After elution of the mobile components, the tightly adsorbing components remaining at the top of the column were eluted with methanol.

TLC. Thin-layer chromatography (TLC) was performed with 0.25-mm silica gel plates (catalog no. 5715; Merck Kieselgel). All of the chromatograms shown were developed with CEE solvent and sprayed with vanillin reagent to visualize the separated components.

Vanillin staining. After evaporation of the developing solvent, TLC plates were sprayed with a freshly prepared solution of 3% vanillin in absolute ethanol containing 1% H_2SO_4 and were heated at 120°C for 5 min to develop the color. psi-active components stained deep blue initially and turned gray after a few days.

Acidic solvents. Reactions of certain psi components were performed in acidic solvents, which were prepared by adding 10 μl of concentrated HCl to 5 ml of the solvent (either ethyl acetate or an alcohol).

Mass spectrometry. Mass spectrometric analysis was performed by using a Finnigan 8230 spectrometer with a magnetic sector. Samples were analyzed by direct chemical ionization with isobutane as the ionizing gas.

RESULTS

Response of the mycelial lawn to psi factor. The reproductive cycle of *A. nidulans* is characterized by two temporally distinct modes of sporulation. In the first mode, green asexual spores (conidia) are formed by mitosis on aerial stalks, while in the second, later-occurring-mode sexual spores (ascospores) are formed by meiosis inside of closed spherical shells called cleistothecia.

The original defining property of psi factor was its ability to induce premature sexual sporulation and to inhibit asexual sporulation. This response is shown in Fig. 1A, in which the inner light zone around the assay disk consists of cleistothecial primordia and the outer dark zone is the green conidial lawn. Although visually striking when optimal, this response is not an ideal assay of activity for purposes of purification, since it requires 2 to 3 days and since the conidial lawn often fails to develop uniformly.

A second manifestation of psi activity is the formation around the assay disk of a yellow-pigmented plaque which is observable on the back of the assay plate (dark area in Fig. 1B) and is optimally detectable from 18 to 24 h after inoculation. Because this back-side response is more rapid and more reliable than the front-side response, it was used as the standard assay for psi activity in the fractionations described below. The two responses could, of course, be due to separate activities, but in fact they always fractionated together. The chemical nature of the yellow pigment is unknown; it does not diffuse into the agar, and attempts to extract it from the mycelium with a variety of solvents have not been successful.

Purification of two psi-active components. TLC analysis of a 20-fold-enriched LHP extract, prepared as described in Materials and Methods, showed three distinct zones of psi activity (A, B, and C in Fig. 2). Examination of such TLC plates with UV light revealed a number of absorbing or

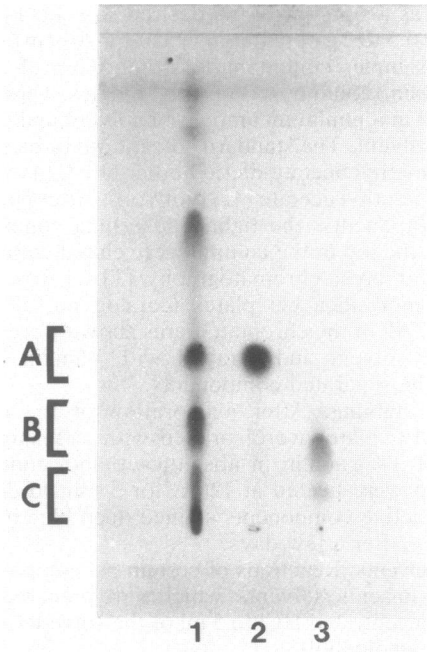

FIG. 2. TLC of purified psiA1 and psiB1. The amount of psiB1 applied to lane 3 was a 10-fold greater fraction of the total yield than the amount of psiA1 applied to lane 2. The plate was stained with vanillin reagent, as were all TLC plates shown in subsequent figures. The brackets indicate the location of psi activity determined by assay of excised zones of an identical chromatogram of the LHP extract. Lanes: 1, LHP extract (20-fold purified); 2, purified psiA1; 3, purified psiB1.

fluorescing components, but none were clearly coincident with the psi-active zones. At this stage it appeared that either the psi components have no UV chromophore or else psi activity resides in components too minor to be detected visually.

In the course of screening numerous chromogenic indicators, we found that vanillin, in the presence of sulfuric acid, formed dark blue reaction products closely coincident with the three psi-active zones (Fig. 2, lane 1). The reactivity of psi-active components with vanillin was confirmed by further fractionations. Although reputedly not very specific, vanillin reacts with only a small minority of the metabolites of *Aspergillus* extracts observable by other means. The active components in these three zones were designated generically psiA, psiB, and psiC; isolated components were given numerical suffixes to allow for possible additional components in the same zone.

The apparently single component in zone A was purified by chromatography through a column of silica gel with CEE as the eluting solvent. Impurities, visible on TLC plates by UV or by vanillin or iodine staining, were eliminated by repeated chromatography with the same solvent system and with the solvent benzene-ethyl acetate (4:6 [vol/vol]). The final product, psiA1 (Fig. 2, lane 2), was a colorless, odorless oil with a yield of about 500 μg per liter, which is about 0.4% of the starting material. Examination of this material by TLC using several different solvent systems showed a single vanillin-staining spot which was completely coincident with activity. A second, minor activity (psiA2) was resolved from psiA1 by the silica gel column but was not further purified.

The amount of extract applied to lane 1 of Fig. 2 corresponded to 60 ml of culture medium. When the same volume

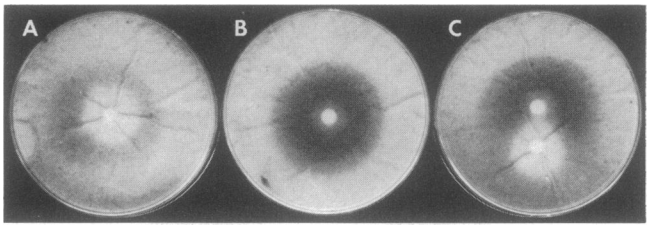

FIG. 3. Different responses to psiA1 (A) and psiB1 (B) and both (C). The psiA1 disks contained 7 μg, while the psiB1 disks contained one-tenth of this amount. Shown are the backs of the assay plates after incubation at 37°C for 24 h.

of uninoculated medium was extracted and analyzed, no trace of a component corresponding to psiA1 was seen, showing that this compound is, in fact, an *Aspergillus* metabolite.

A major active component present in zone B of Fig. 2 was purified by methods similar to those used for psiA1. In this case, however, the activity could not be eluted from a silica gel column with the standard CEE solvent but could be eluted with pure ethyl acetate. Purification was achieved in repeated runs with increasing proportions of ethyl acetate in a chloroform-based solvent system. The purified product (psiB1), shown in lane 3 of Fig. 2, was also an oil.

The yield of psiB1 (20 μg/liter, which is 0.02% of the starting material) was some 25-fold less than that of psiA1, but the specific activity was considerably higher. The threshold for detectability of psiA1 activity is about 200 ng, whereas for psiB1 it is about 25 ng. Spectral analysis, described below, indicated that psiA1 and psiB1 are probably closely related structurally.

The highly polar components in zone C included pigmented compounds which, together with psi-active components, tightly adsorbed to silica gel columns and could be eluted only with methanol. We have not yet been able to obtain pure psi-active components from zone C.

Biological activity of psiA1. With the separation of several psi components, it became evident that the biological activity of psiA1 is qualitatively different from that of psiB and psiC. In the standard assay, psiB and psiC produced a homogeneous yellow plaque on the back of the assay plate (Fig. 3B), whereas the pigmentation zone produced by psiA1 was less intense and was halo shaped (Fig. 3A) because of the absence of pigment in the zone near the assay disk. Furthermore, when a psiA1 assay disk was placed close to a psiB1 assay disk on the same plate, psiA1 produced a nonpigmented island in the otherwise uniform plaque centered on the psiB1 disk (Fig. 3C). It thus appears that, at high concentration near the assay disk, psiA1 is antagonistic to the response elicited by psiB1, which will be referred to as the agonistic response. The amount of psiB1 applied to the disks in Fig. 3 was one-tenth of the amount of psiA1. However, no halo was seen for psiB1 when 20 times more sample was applied.

psiA1 reacts with alcohols. In early attempts to purify psi using activity alone as an assay, chromatographic fractionations were often inexplicably irreproducible. At least part of the reason for this irregular behavior was revealed when a sample of psiA1 was eluted from a TLC plate, concentrated by evaporation of the solvent, and rechromatographed. The major vanillin-reactive component of the eluted sample then had an R_f considerably lower than that of psiA1.

The origin of this new component was traced to the use of methanol as the eluting solvent. Elution with a nonalcoholic

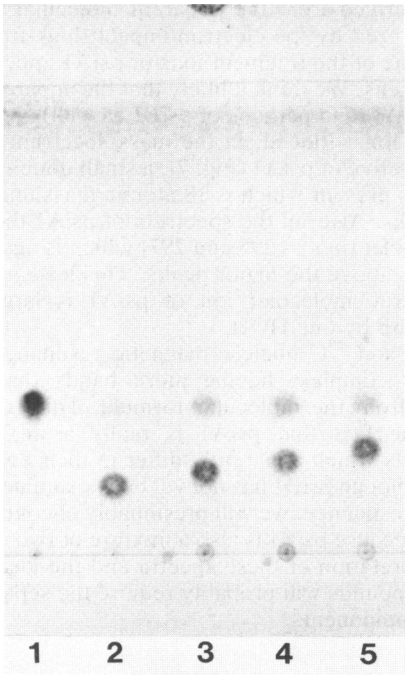

FIG. 4. Reaction of psiA1 with alcohols. The reaction mixture consisted of 15 μg of psiA1 dissolved in 50 μl of the indicated acidic alcohol. After being heated at 40°C for 1 h in a capped vial, the samples were evaporated and applied to the TLC plate in ethyl acetate. Lanes: 1, unreacted psiA1; 2, psiA1 reacted with acidic methanol; 3, psiA1 reacted with acidic ethanol; 4, psiA1 reacted with acidic 2-propanol; 5, psiA1 reacted with acidic 1-butanol.

solvent yielded only psiA1, while incubation of psiA1 with weakly acidic methanol produced efficient conversion to the lower-R_f component (Fig. 4, lane 2). Furthermore, psiA1 was shown to react with ethanol, 2-propanol, and 1-butanol (Fig. 4, lanes 3, 4, and 5), yielding a series of chromatographically distinguishable products. Conversion was practically nil unless acid was present. The initial inadvertent conversion was probably due to HCl originating from chloroform, which had been used as a solvent in prior fractionations of this sample.

To determine whether the reaction products of psiA1 with alcohols retain psi activity, the derivatives were purified by elution from a TLC plate. Assays for psi activity showed that in each case the derivatives produced a more intense psi response than psiA1 itself and that they lacked the antagonistic activity of psiA1. The movement of psi activity on the TLC plate from the psiA1 site to the site of the alcohol derivative is strong evidence that psi activity resides with the alcohol-reacting compound rather than with some minor component that copurifies with psiA1.

Reactions of alcohols with organic compounds are generally reversible, and this is true for the reaction of psiA1 with alcohols, as shown for butanol in Fig. 5. Heat alone produced significant reversion of the butanolic reaction product to psiA1 (Fig. 5, lane 3), and almost total reversion was obtained by incubating the product in acidic ethyl acetate (Fig. 5, lane 4). Furthermore, in the presence of methanol, the butanolic product very efficiently interconverted to the methanolic product (Fig. 5, lane 5). This reaction is indicative of a transesterification in which one alcohol replaces another.

psiB1 showed no apparent reaction with alcohols and was not converted to psiA1 in an acidic nonalcoholic solvent.

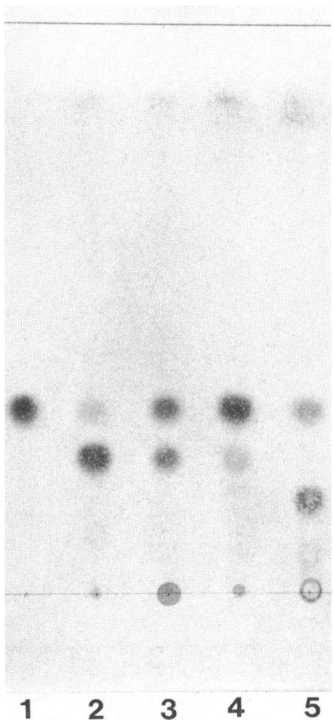

FIG. 5. Reversion and transesterification of the butanolic derivative of psiA1. The butanolic derivative of psiA1 was prepared as described in the legend to Fig. 4. Samples of this derivative were used for the reactions of lanes 3 to 5. Lanes: 1, unreacted psiA1; 2, butanolic derivative of psiA1; 3, butanolic derivative of psiA1 heated on plate for 30 min at 90°C; 4, butanolic derivative of psiA1 heated in acidic ethyl acetate for 1 h at 40°C; 5, butanolic derivative of psiA1 heated in acidic methanol for 1 h at 40°C.

psiA1 can be generated from a zone C component. The material present in zone B of Fig. 2 includes, in addition to psiB1, at least one component that can be converted to psiA1 by incubation with acidic ethyl acetate. This is not surprising, because this LHP extract had been in contact with methanol and chloroform during purification and the methanolic derivative is located in zone B. Moreover, chloroform, in addition to being acidic, contains about 1% ethanol added as a preservative.

To determine whether there exist natural components convertible to psiA1, we processed an extract to the approximate purity of the LHP extract of Fig. 2, but without the use of any alcohol (see Materials and Methods). Zones B and C were eluted, incubated with acidic ethyl acetate, and rechromatographed to detect the presence of psiA1. psiA1 was generated from the psiC material (Fig. 6). Furthermore, when acidic butanol was used as the solvent for the reaction, the butanolic derivative of psiA1 was formed even more efficiently than psiA1. No detectable psiA1 was generated from the zone B material (not shown), leading us to suspect that, for preparations which had been in contact with methanol or chloroform, some of the activity of zone B was an artifact of purification.

Spectrometric analysis of psi species. Mass spectrometric analysis of psiA1 indicated an apparent molecular ion of $m/e = 276$ with the composition $C_{18}H_{28}O_2$. The mass required for this composition is 276.2090; the measured mass was 276.2089. In several independent preparations this ion was accompanied by a strong M + 2 peak. The methanolic and

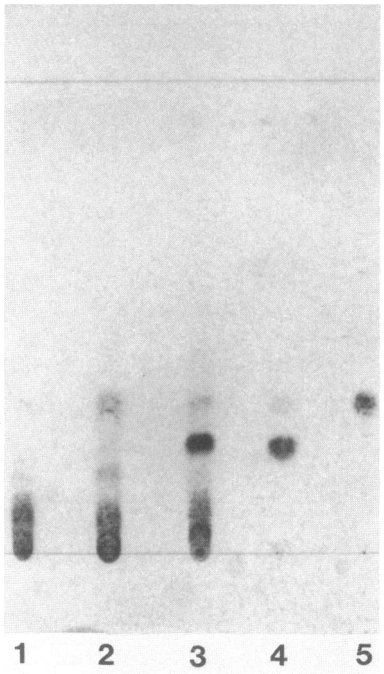

FIG. 6. Generation of psiA1 from psiC. psiC was prepared by elution of the zone C material from a TLC plate such as that shown in Fig. 2 except that the extract was prepared without the use of any alcohol. The samples of lanes 2 and 3 were incubated in the indicated solvent at 40°C for 1 h. Lanes: 1, unreacted psiC; 2, psiC heated in acidic ethyl acetate; 3, psiC heated in acidic 1-butanol; 4, butanolic derivative of psiA1; 5, unreacted psiA1.

butanolic derivatives of psiA1 showed apparent molecular ions of m/e = 308 and m/e = 350, respectively, indicating the addition of an entire molecule of the respective alcohol (Fig. 7). The main reaction of psiA1 with alcohols thus appears to be not a replacement solvolysis reaction but rather an addition reaction, such as the opening of a lactone ring to form a hydroxy ester or the formation of a hemiacetal. These alcoholic derivatives of psiA1 also gave strong M + 2 peaks, showing that both species of psiA1 react with alcohols. The two species were designated psiA1α (m/e = 276) and psiA1β (m/e = 278).

psiB1 showed an apparent molecular ion of m/e = 278 with the composition $C_{18}H_{30}O_2$ (required mass, 278.22458; measured mass, 278.22464) and again a strong M + 2 peak. These two species were designated psiB1α (m/e = 278) and psiB1β (m/e = 280). The closely similar compositions of psiA1 and psiB1 and the occurrence of the M + 2 component in both suggests that these compounds are closely related.

The infrared spectrum of psiA1 showed a strong carbonyl absorption band at 1732 cm^{-1} and a strong C-O stretch band at 1250 cm^{-1}, which is consistent with the presence of an ester group. If the alcoholic derivative of psiA1 is a hemiacetal, the carbonyl absorption of psiA should vanish for the derivative. We found, however, that the methanolic derivative retained the carbonyl absorption. Since the only other likely reaction with an alcohol is the opening of a lactone ring, these results strongly suggest that psiA1 is a lactone.

The infrared spectrum of psiA1 also had a strong absorption at 3430 cm^{-1}, which is indicative of a hydroxyl group. If psiA1 is an ester, which accounts for two oxygens, the presence also of a hydroxyl group implies that the molecule has three oxygens, which is inconsistent with the molecular formula derived from the apparent molecular ion. In fact, when analyzed by the electron-impact (high-fragmentation) mode, some of the fragment ions of psiA1 appeared to have three oxygens. We think it likely that the apparent molecular ions of psiA1 (and perhaps of psiB1 as well) are not the true molecular ions. Indeed, in the mass spectrum of the butanolic derivative of psiA1 (Fig. 7), a small doublet (m/e = 369 and 371) is present which is 18 atomic mass units above the major peaks. Also, in the spectrum of psiA1 there is a very minor doublet (m/e = 295 and 297) which is again 18 atomic mass units above the major peaks. This leads us to suspect that the true molecular ion of psiA1 is larger than the apparent ion by one HOH.

The ^1H and ^{13}C nuclear magnetic resonance spectra of psiA1 are complex, having more bands than would be expected from the molecular formula. This is presumably due to the fact that psiA1 is really a mixture of two components which apparently differ in their degree of saturation. Although psiB1 has not yet been examined by nuclear magnetic resonance, we will presumably encounter the same problem because psiB1 is also a mixture of two components. The interpretation of these spectra and the identification of these compounds will probably require the separation of the α and β components.

DISCUSSION

Using the vanillin chromogenic reagent combined with activity assays, we have purified two of the most abundant psi-active components, psiA1 and psiB1, and have found that each consists of two species which differ in composition by two hydrogen atoms. The fact that all four components have very similar compositions suggests that they are structurally related, but this has yet to be proven. The properties and nomenclature of the various psi components are summarized in Table 1.

The biological activity of psiA1 is curious in that, unlike the other psi components, it exhibits both agonistic and antagonistic activity, resulting in a halo-shaped response on the assay plate (Fig. 3). This behavior can be understood if the intrinsic activity of psiA1 is totally antagonistic and if psiA1 is converted into agonistic derivatives by the mycelium on the assay plate. Thus, the agonistic response to psiA1 is observed distal from the assay disk, where the concentration of residual psiA1 is low. The conversion could occur by reaction of psiA1 with endogenous hydroxy compounds, as occurs in vitro with exogenous alcohols. In support of this hypothesis is our demonstration that at least one component, located in zone C of Fig. 2 (prepared without the use of any exogenous alcohol), can undergo acid-catalyzed conversion to psiA1, thus establishing a structural, if not a metabolic, relationship between these two components. This observation leads us to suggest that sexual sporulation in *A. nidulans* may be triggered by the occurrence of reactions that convert antagonistic psiA1 into agonistic psi components. If this is the case, however, the conversions must involve more than alcoholic esterification because the composition of psiB1 is not consistent with the mere addition of an alcohol to psiA1.

The reactivity of psiA1 with alcohols also suggests a mechanism by which psi components might interact with fungal tissue. If, as argued above, the activation of psiA1 involves alcoholic esterification, further reaction with a hydroxy-containing membrane component, such as a sterol, could occur by transesterification, which has been shown to be a very efficient reaction in vitro (Fig. 5). Presumably,

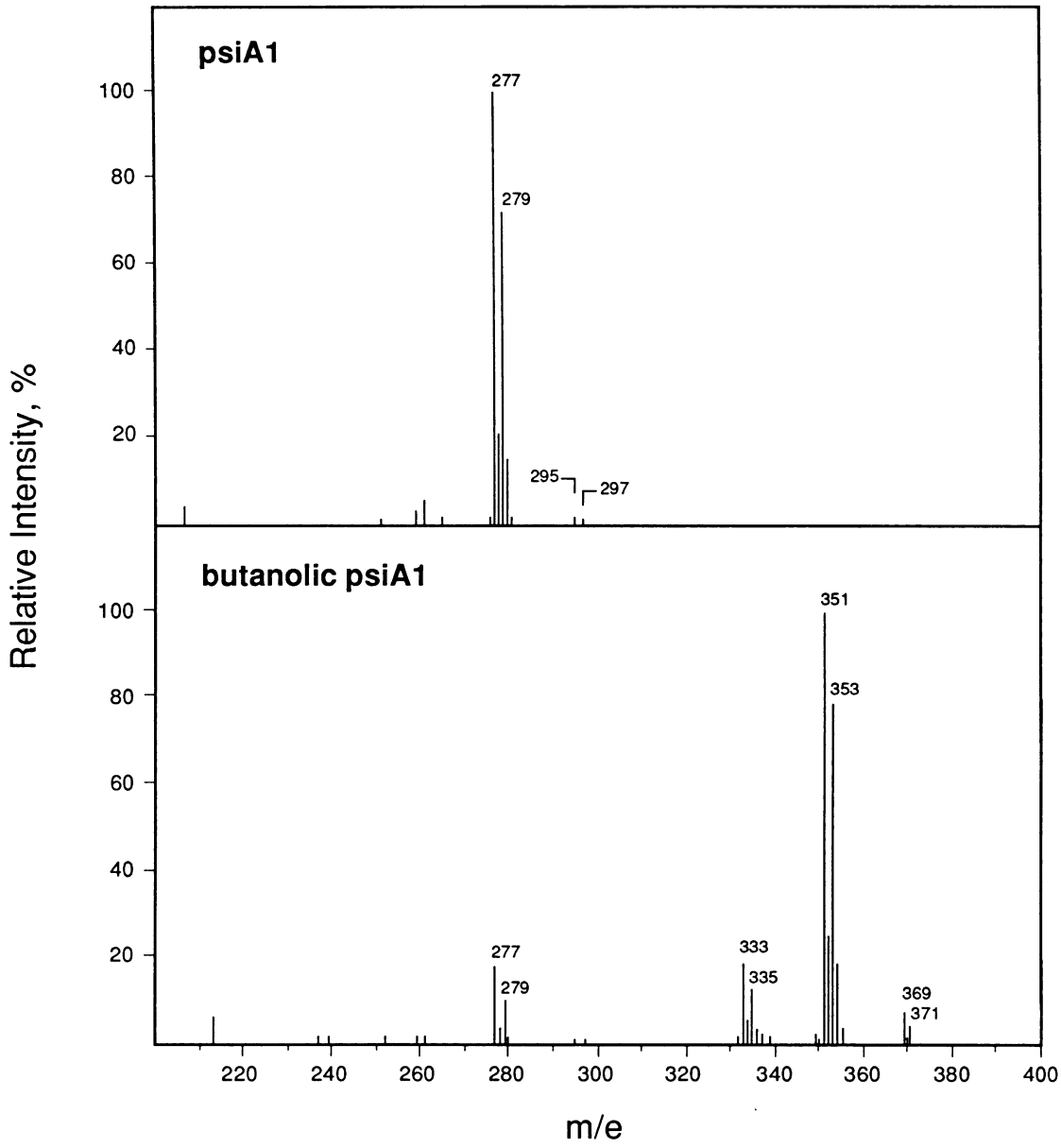

FIG. 7. Mass spectrometric analysis of psiA1 and its butanolic derivative. Samples were analyzed by direct chemical ionization, which gives ions 1 atomic mass unit greater than the true ion. Thus, the base peaks (highest intensity) are *m/e* = 276 for psiA1 and *m/e* = 350 for the butanolic derivative.

psiA1 could react directly with sterols in vitro, but the in vivo reactions, unlike the acid-catalyzed reactions, may be governed by enzymatic specificity. Although we have argued otherwise, the alcohol reactivity of psiA1 may be an incidental property of this compound with no relevance to *Aspergillus* development. An analysis of the fate of labeled psiA1 when fed to actively developing fungal tissue should help to decide the question.

The nonpeptidyl fungal sex hormones that have been most extensively characterized are the steroid antheridiol ($C_{29}H_{42}O_5$, 470 daltons) of *Achlya* species and the terpene trisporic acid ($C_{18}H_{26}O_4$, 306 daltons) of *Mucor* species (4). Antheridiol is present in culture medium in extremely small amounts (10^{-8} M), and a concentration as low as 10^{-11} M can be detected by bioassay. By contrast, the yield of trisporic acid is about 10^{-6} M, and the limit of the bioassay is 10^{-8} M. The yield of psiA1 that we obtained in our purification (0.5 mg/liter [2×10^{-6} M]) is quite comparable with that of trisporic acid. However, the limit of detection by bioassay for the most active isolated form of psi (psiB1) appears to be some 10-fold higher than for trisporic acid (assuming that, at the detection limit in our assay, the compound has diffused into about 1 ml of agar).

The hormones of heterothallic species actually exist as conjugate pairs in which each member of the pair is specific to one of the two mating types. For example, neither of the two mating types of *Mucor* species alone produces trisporic acid, but rather they produce mating-type-specific prohormones which are converted to trisporic acid by mycelia of the opposite mating type in mixed cultures (4). *A. nidulans* is

TABLE 1. Summary of psi component properties

Component(s)	Mol wt	Formula	Yield (μg/liter)	Activity	Reactivity
psiA					
psiA1α	294	$C_{18}H_{30}O_3$[a]	500[b]	Agonistic and antagonistic	Reacts reversibly with alcohols
psiA1β	296	$C_{18}H_{32}O_3$[a]		Agonistic and antagonistic	Reacts reversibly with alcohols
psiA2	?	?	?	Agonistic	?
psiB					
psiB1α	278	$C_{18}H_{30}O_2$	20[b]	Agonistic	No reaction
psiB1β	280	$C_{18}H_{32}O_2$		Agonistic	No reaction
Alcoholic artifacts				Agonistic	Converts to psiA1
psiC	?	?	?	Agonistic	Converts to psiA1

[a] Formulae for psiA1 species are based on the assumption that the true molecular ions have one HOH more than the major ions seen by mass spectrometry (see Results).
[b] Combined α and β components.

homothallic, but the basis of this homothallism is unknown. The mechanism could be similar to that in *S. cerevisiae*, in which, because of rapid mating type interconversion, homothallic strains are actually mixtures of the two mating types (5). We thus might suspect (by analogy with heterothallic systems) that some of the components of psi factor are conjugate pairs. Presumptive heterothallic species of the *A. nidulans* group have been isolated (7). An interesting question to be explored is whether the heterothallic and homothallic species differ with respect to their spectra of psi components.

The source of the extracts used in the present study was a mutant of *A. nidulans* that overproduces psi activity together with a variety of phenolic metabolites. This mutant is totally asporogenous, forming neither sexual spores, asexual spores, nor spore-bearing structures. Several nonallelic thermosensitive mutants of this class have been isolated and, from temperature-shift experiments, all appear to be blocked at a very early stage of the sporulation process (1, 2, 9). To explain the phenotype of these mutants we had previously postulated that the mutational block is in a metabolic pathway whose end products are hormones that induce both sexual and asexual sporulation and that the compounds responsible for psi activity are accumulated upstream intermediates of this pathway (3). The mutants would be asporogenous because they lack both of the hormones. The wild type, which is used in the psi assay, would respond to the added intermediates because it can complete the synthesis of the hormones. This model predicts that the spectrum of psi-active components of the mutants should be different from that of the wild type and may be different from each other. With the ability to visualize and isolate psi-active components, we are now in a position to test these possibilities directly.

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